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USE OF THE POLYMERASE CHAIN REACTION
IN THE DIAGNOSIS OF
BOVINE LEUKOSIS

by

Emma Jane Kelly

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Bioveterinary Science

UTAH STATE UNIVERSITY
Logan, Utah

1992

DEDICATION

I am dedicating this thesis to my parents, Susan and Stephen Kelly. They have been very supportive throughout my academic career and taught me the value of hard work and dedication in the successful completion of a project. At times I felt overwhelmed with working full-time, being on call, taking classes and doing research, but with their support I finished this degree.

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I wish to thank Dr. Rob Callan for putting the idea into my head to work with PCR, and for his support and enthusiasm. I am grateful to Dr. Jay Call for being very flexible and giving me time off for coursework while I was working for him as the clinical veterinary resident. Thanks are also due to my other committee members, Dr. Morrey and Dr. Barnett, for their helpful comments while finalizing this thesis.

Last, but certainly not least, I will be eternally grateful to my fiance, Paul Dawson, for his help and patience at all stages of this project. At times, I think I would have gone crazy without him.

Emma Jane Kelly

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ABSTRACT

Use of the Polymerase Chain Reaction
in the Detection of
Bovine Leukosis

by

Emma Jane Kelly, Master of Science
Utah State University, 1992

Major Professor: Dr. M. Keven Jackson
Department: Animal, Dairy, and Veterinary Sciences

A diagnostic test for bovine leukosis was developed using the polymerase chain reaction (PCR) to amplify a 375 base pair region in the gag gene of the proviral genome.

Blood samples were collected from 3 adult Holstein cows shown to be infected with bovine leukosis virus (BLV) by the agar-gel immunodiffusion (AGID) technique. The 3 samples were mixed and the composite blood was used to inoculate 10 cows. Five of the cows were inoculated with 0.1 ml of blood, and the other cows were inoculated with 1 ml of blood. Five of the cows were negative for BLV by AGID and PCR on the day of inoculation, and the other five cows were positive for BLV on the day of inoculation.

The 10 cows were bled on day 1 (day 0 being the day of inoculation) and day 7 post-inoculation, and every 2 weeks subsequently until 3 months post-inoculation. Samples were stored until the end of the study, at which time the AGID and

PCR tests were performed.

The 5 cows that were positive on day 0 remained positive by AGID and PCR for the remainder of the study.

Three cows became AGID-positive 3 weeks post-inoculation, and two cows seroconverted 5 weeks post-inoculation. The time of seroconversion did not correlate with the volume of viral inoculum. In comparison, the PCR consistently detected infection sooner than the AGID: by day 7 post-inoculation all 5 cows were BLV-positive as determined with the PCR test, and remained positive until the end of the study.

The results indicate that under the experimental conditions, bovine leukemia virus infection in cattle can be detected as much as 2 to 4 weeks earlier by PCR than by AGID.

(84 pages)

INTRODUCTION

The purpose of this research was to develop a diagnostic test for bovine leukosis virus (BLV) using the polymerase chain reaction (PCR), then to compare the PCR with agar-gel immunodiffusion (AGID). The hypothesis was that the PCR would be more sensitive and specific than AGID.

The ideal diagnostic test to detect BLV infection would have the following properties:

1. High sensitivity and specificity. A test that would detect the actual virus or antigen would be preferable since AGID detects anti-BLV antibodies.
2. A quick and easy procedure that could be applicable to large scale BLV detection.
3. Relatively inexpensive.
4. Able to distinguish colostral antibody from that acquired by natural infection. A test to detect the actual virus would be preferable since young animals with colostral antibody are not infected with the virus, and would, therefore, not give false positive test results.
5. Able to detect antibody or antigen in milk, including bulk samples from herds with a low BLV prevalence.

A test using the polymerase chain reaction (PCR) was developed to detect BLV infection. PCR was utilized to amplify a specific DNA sequence of the bovine leukosis provirus for subsequent detection by gel electrophoresis. This procedure has the potential to be a very sensitive test, able to be performed on blood or milk, and highly specific for BLV with

no false positives due to colostral antibody.

The radioimmunoassay (RIA) and enzyme-linked immunoabsorbent assay (ELISA) tests are very specific. However, the RIA is not quick and easy to perform and the ELISA, though easy to perform, may detect a trace of positive contaminating serum in a negative sample. Neither test can differentiate colostral antibody from natural infection, nor detect antibody in bulk tank milk. The AGID test is quick, easy and inexpensive, but lacks sensitivity, cannot distinguish colostral antibody and cannot detect antibody in milk, as a rule. The other serologic tests mentioned in passing have not been sufficiently used and evaluated. The current methods used to detect the actual virus generally use tissue culture, which is expensive and laborious.

REVIEW OF LITERATURE

Bovine Leukosis

Bovine leukosis has been described in the literature for many years. The first documented case was in Germany in 1871 (1). Most early descriptions of the disease were in Europe, though several cases occurred in the United States.

Forms of the Disease

There are 2 forms of the disease: enzootic bovine leukosis and sporadic bovine leukosis. Sporadic bovine leukosis is uncommon and affects animals under 3 years of age (2). There are 3 forms of sporadic bovine leukosis: the juvenile form that affects calves less than 6 months old, the thymic form in animals less than 2 years of age, and the cutaneous form in animals of 1-3 years of age (2). The cause of sporadic bovine leukosis is unknown. The more common form is enzootic bovine leukosis (or adult multicentric leukosis) in which disease occurs in adult animals. The etiologic agent of enzootic bovine leukosis is bovine leukemia virus (BLV) in the family Retroviridae, subfamily Oncovirinae (3,4). Development of clinical disease is rare. Persistent lymphocytosis is a benign reaction to BLV (2); cattle may later develop lymphosarcoma or may remain nonclinical for the remainder of their lives. Persistent lymphocytosis is not a precancerous condition.

Prevalence

Enzootic bovine leukosis is present worldwide, with some areas having a higher prevalence, such as parts of South America, North America, Asia, and much of Europe. It is estimated that at least 20% of the adult dairy cattle in the USA are seropositive to BLV (2). However, there are very few BLV-positive cattle in the United Kingdom, New Zealand, and Australia (2). Infection in beef cattle is much less common (approximately 1-20%) (4). Mortality due to the disease is quite low (2-5%) (2).

Species Affected

In nature, cattle are the only host species for BLV (2,4). Experimentally, the virus has been transmitted to sheep (5,6,7,9,10,11,12), goats (8), and chimpanzees (13). BLV can infect cats, dogs, and rabbits (14). It was concluded (15) that experimental animals (mice, guinea-pigs, and hamsters) are not suitable for studies of bovine leukosis because infection with BLV in these animals produced no characteristic lesions. Attempts to transmit it to swine (16) have been unsuccessful. Olson and Driscoll (17) examined the relationship between cases of human and bovine leukemia to see if there is a correlation between the two. They found that human beings in close contact with potentially BLV-infected cattle do not develop antibodies to BLV (17). In a similar study, sera from humans likely to be exposed to BLV (animal

caretakers, laboratory personnel, veterinarians) were tested for BLV (18): no specific antibodies to BLV were found. However, the authors reported that BLV does grow in human cells. Another study was performed (19) to see if there was a relationship between human acute lymphoid leukemia in 223 people in Iowa and exposure to dairy cattle and drinking of raw milk. The authors concluded that there was no relationship.

Nature of the Virus

The virus is present in host B lymphocytes (2) and its RNA genome is copied into a DNA molecule by a viral enzyme called reverse transcriptase (RNA-dependent DNA-polymerase) (4). This DNA enters the host cell nucleus and becomes permanently integrated into the host cell DNA, and is, therefore, multiplied as host cell division occurs (2,4). The viral infection is permanent because of its location within cells (2). The virus does not need to multiply for survival or transmission because it is permanently integrated in the B lymphocytes, so that when the cell divides the viral DNA is present in the daughter cells. The infected animal sheds infected cells even though it may develop specific antibody (2,4). Bovine leukemia virus is a retrovirus, in the subfamily *Oncovirinae* (20). The genome of the virus includes 3 genes (20):

1. The *gag* gene (group-specific antigen) which encodes the internal structural virion core proteins known as p15, p24,

and p12.

2. The *pol* gene (polymerase) which encodes the reverse transcriptase enzyme.

3. The *env* gene coding for the virion envelope glycoproteins (envelope) known as gp60 and gp51.

The virus is a C-type particle, by virtue of morphology of the virus seen within infected cells by electron microscopy (20). Retroviruses are RNA viruses with a linear single-stranded genome (20). Other viruses in the subfamily *Oncovirinae* affect chicken, swine, and cats (20). The retrovirus family also includes 2 other subfamilies, the *Lentivirinae* and the *Spumavirinae* (20). The lentiviruses include Bovine Immunodeficiency Virus (BIV), as well as the causative agent of human AIDS. The spumaviruses are nonpathogenic (20).

Rice, Stephens, and Gilden (21) sequenced a tumor-derived bovine leukemia provirus and summarized the BLV genome. From the 5' end, the BLV genome can be represented as follows (21):

R+U5--GAG--POL--ENV--pX--U3+R

1. R represents a sequence of nucleotides repeated at the 5' and 3' extremities of the viral RNA.
2. U5 and U3 are sequences unique to the 5' and 3' end of the genome, respectively.
3. The long terminal repeat is a combination of RU5 and U3R.
4. pX is the region between the 3' end of the *env* gene and the

5' end of U3. It is a region of 1817 nucleotides which encodes genes regulating virus expression.

Response to Viral Exposure

Upon exposure to virus, some animals do not become infected, some animals become permanently infected and form antibodies (latent carriers), while others become permanently infected, form antibodies, and develop persistent lymphocytosis (2). Finally, the minority of the exposed animals become infected, seroconvert, may or may not have had persistent lymphocytosis, and develop lymphosarcoma (2). The incidence of neoplasia is very low, 5% or less (2).

It appears that the outcome of infection may be genetically determined (22). Therefore, an individual animal's response to exposure to the virus may depend on its level of genetic resistance or susceptibility, as well as the infective dose of virus, the virulence of the virus, and the animal's immune status.

Experimentally, the infection first spreads to the spleen (virus recoverable 8 days post-infection), then virus is present in peripheral blood leukocytes a week later (2 weeks post-infection), and antibodies are detectable in serum 5-6 weeks after infection (23). In this experiment, calves of approximately 15 months of age and negative for antibodies to BLV were inoculated subcutaneously with approximately 250,000 lymphocytes from a BLV positive steer. In natural infections, seroconversion occurs 3-4 months after a negative adult animal

is placed in a positive group (2). Passive (or colostral) immunity persists for 2-7 months (2).

Economic Loss Associated with Disease

Though enzootic bovine leukosis is associated with a very low mortality, there is considerable economic loss due to the disease. Direct losses associated with the virus include decreased milk production and condemnations at the slaughter house (24). These losses are minor compared to the indirect losses due to the political and social stigma associated with the disease (24). Many countries without the disease will not import cattle without a negative blood test (2). In the United States, approximately 44 million dollars is a conservative estimate of the annual cost of the disease (24). Death losses due to lymphosarcoma will be more significant in certain dairy herds that keep pedigreed animals or seedstock. These pedigreed animals are more valuable than commercial animals and have a higher average age.

Transmission of the Virus

Transmission of the virus is mainly horizontal via infected lymphocytes, such as in blood, milk, and tumor masses (2). BLV has also been detected in nasal and tracheal washings (25). In utero transmission occurs, but is much less significant (26). Jacobsen et al. (26) found that 4.7% of calves in one herd and 3.4% of calves in another herd born to BLV-seropositive cows were seropositive at birth. This indicates a low incidence of

in utero BLV transmission. An earlier study (27) reported that 18% of calves born to BLV-positive cows were BLV-positive before they received colostrum. The authors (27) found that most of the cattle that became infected with BLV did so after exposure to the adult herd. During their early life, they were protected against horizontal transmission by colostral antibody (27). Natural transmission seems to require close contact between infected and susceptible animals for a prolonged period of time in order for infected lymphocytes to enter the skin of the susceptible animal (4). Insects have been shown to transmit the virus (28,29,30,31). Iatrogenic transmission of the virus has occurred via blood associated with husbandry and veterinary practices such as gouge dehorning (32), ear tattooing (33), needles (34,35), and rectal palpation (36,37). It is felt that calves are susceptible to BLV infection via milk if they are born to a BLV-negative dam and, therefore, are not protected by colostral antibody. However, a recent preliminary study (38) suggests that the dose of BLV-infected lymphocytes normally present in milk, which is considerably less than in an equal volume of blood, may be insufficient to infect calves. It has been shown experimentally (39) that calves born to BLV positive cows are protected from infection by colostral antibodies. This is presuming that the calf did not get BLV by in utero transmission and receives colostrum before horizontal exposure to the virus. Semen, embryos used in embryo

transfer, saliva, urine, and feces do not appear to play a role in transmission of the virus (40). Another report (41) demonstrated the noninfectivity of semen from infected bulls. An experiment (42) in which calves from BLV-positive dams and BLV-negative dams were transplanted as embryos into both BLV-positive and negative recipients reconfirmed that transmission of BLV by embryo transfer occurs rarely or never.

Inactivation by Pasteurization

Pasteurization does inactivate the virus (43,44,45). Roberts et al (43) added 10^7 BLV-infected lymphocytes to 500 ml of milk pooled from BLV-free cows, and heat-treated the milk at temperatures from 40°C to 65°C for 70 seconds. Sheep were then inoculated with the heat-treated milk and tested for BLV by the AGID test every month for 6 months. At the end of the 6 months, virus isolation of BLV was attempted in all of the sheep. The researchers (43) found that BLV infectivity in infected lymphocytes was destroyed when the milk was heated at 50°C or above for 70 seconds. This high-temperature short-time procedure is similar to commercial pasteurization (43). Rubino et al (44) studied the survivability of BLV-infected lymphocytes in milk. They found that BLV in milk can survive at least 3 days of refrigeration temperatures, and that variations in milk constituents such as fat, protein, total solids, and cells had no significant effects on BLV survivability (44). However, pasteurization at 63°C for 30

minutes did inactivate the BLV-infected lymphocytes (44). The conclusions of a study in Australia (45) were that Australian commercial pasteurization procedures (63°C for 30 minutes or 72° to 73°C for 15 to 20 seconds) are sufficient to destroy the infectivity of BLV and BLV-infected lymphocytes in milk.

Diagnosis and Clinical Disease

Confirmation of BLV infection has been made by demonstrating persistent lymphocytosis, by confirming development of lymphosarcoma, by virus isolation, or by serology.

Lymphocytosis is an increase in the total white blood cell (WBC) count. The normal bovine WBC count ranges from 6,000 cells/microliter to as high as 15,000 cells/microliter, and the percentage of lymphocytes is approximately 60%. Cows with persistent lymphocytosis have a greatly increased percentage of lymphocytes, especially immature forms (2), as well as an elevated total WBC count. Before the viral etiology of the disease was known, persistent lymphocytosis was used to diagnose bovine leukosis. Lymphocyte "keys" correlating total number of leukocytes and percentage of lymphocytes were used to assess the degree of suspicion for leukosis (46). Currently, much less emphasis is placed on this finding.

If neoplasia develops, depending on its location, clinical findings may provide a presumptive diagnosis. Sometimes clinical signs are very nonspecific, such as decreased milk

production and decreased appetite. Unilateral or bilateral exophthalmus may be seen with retrobulbar tumors. Occasionally, the lymphosarcoma will be intraocular (47), in which case there may be redness and epiphora rather than exophthalmus. Lymphosarcoma involving the uterus or pelvis may be palpable on rectal examination. Posterior paralysis unassociated with parturition may be due to spinal leukosis. Lymphadenopathy may be visible if external lymph nodes are involved. Melena and/or sudden death may be due to abomasal ulceration associated with lymphosarcoma. Definitive diagnosis requires histologic examination of a tumor sample obtained by biopsy or necropsy.

In cattle that develop neoplastic disease, the incubation period is usually 4-5 years (2), so that adult cattle between 4 and 8 years of age are most commonly affected (2,4). Lymphosarcoma most commonly develops in the abomasum, heart, visceral lymph nodes, peripheral lymph nodes, and uterus (2,4), though lesions can form in any organ. The course of the disease varies from subacute to chronic (2). Clinical signs depend on the affected organ(s), and include nonspecific as well as specific signs, as already mentioned. In 1100 field cases (48), the clinical signs seen with the highest frequency included weight loss, decreased milk yield, external lymphadenopathy, decreased appetite, internal lymphadenopathy, posterior paresis, fever, respiratory signs, bilateral exophthalmus, diarrhea, constipation, unilateral exophthalmus,

and cardiovascular disease.

Virus isolation requires inoculation of suspect leukocytes onto lamb spleen cells (or other appropriate cells). If there is viral growth, the virus may be identified by electron microscopy, fluorescent antibody test (FAT), enzyme-linked immunoabsorbent assay (ELISA), radioimmunoassay (RIA), or syncytial-infectivity assay (2). Tissue culture work is expensive and laborious, and so serologic diagnosis is usually performed.

Many serologic tests have been described in the literature. These tests rely on the presence of antibody to bovine leukosis present in the animal's blood or milk. Because the virus establishes a permanent infection, there is constant antigen production and antibody response (4). Calves that drink colostrum from BLV-positive cows develop passive antibody titers that persist for 2-7 months (2), so care must be taken in interpreting test results from calves.

Some the serologic tests that have been used to diagnose BLV found in the recent literature are as follows:

1. Agar gel immunodiffusion test (AGID) (3,49,50,51,52).
2. Radioimmunoassay (RIA) (3,49,50,51).
3. Enzyme-linked immunoabsorbent assay (ELISA) (3,53,54).
4. Protein immunoblot test (55).
5. Early polykaryocytosis inhibition test (56).
6. Anticomplement immunofluorescence (3).
7. Complement fixation (3).

8. Indirect immunoperoxidase test (3).
9. Micro counter-current immunoelectrophoresis test (3).
10. Indirect hemagglutination test (3).
11. Chemoluminescence (3).
12. Immunofluorescence, direct and indirect (3).
13. Serum neutralization test (3).

The 3 serologic tests that have been used the most are the AGID, the RIA, and the ELISA. The protein immunoblot test was claimed to be more sensitive than the AGID test (55); however, little other mention has been made of it. The early polykaryocytosis inhibition test is as sensitive as RIA, but requires tissue culture (56). In tests involving complement fixation, there was interference from anti-complement activity of positive BLV sera (3). Immunofluorescence was found to be too laborious for routine work (3).

The AGID test is easy to perform, and has extensively been used as a screening test to diagnose herd infection. It is not considered accurate enough for individual animals because of false positive reactions (2). This is the test recognized by most governments that have cattle importation requirements. Test kits are commercially available. BLV infection can be detected with this test within 2-3 months after virus exposure (4). The disadvantages of the AGID test include the fact that it cannot detect BLV in individual or bulk tank milk samples (52), it cannot distinguish colostral antibody from antibody acquired by natural infection (49), and, as previously

mentioned, there are false positive reactions (2).

There are 2 major antigens of the bovine leukosis virus that are used to detect antibody in suspect sera from cattle and sheep (51). Antigen p24 is a 24,000 dalton, ether-resistant, nonglycosylated polypeptide of the virion core. Antigen gp51 is a 51,000 dalton, ether-sensitive, glycosylated protein of the virion envelope (51). In most animals, the antibody titer to gp51 is higher than the titer to p24, so that immunodiffusion tests must be conducted with gp51 antigen. However, Gauthier et al (56) indicate that some infected cattle react solely with p24 or gp51 antigens, which favors use of a dual antigen. Most commercial AGID testkits have both antigens. AGID is more sensitive than complement fixation and less sensitive than enzyme-linked immunosorbent assay, virus neutralization, and radioimmunoassay (presumably because higher antibody concentrations are required to visualize the precipitation lines in the AGID test than would be necessary to detect antibody by the other immunologic techniques) (51).

The first RIA was developed for p24 (the major internal protein), but there is now an RIA for the major surface glycoprotein (gp51) (51). Gp 51 RIA is the more sensitive test (51). The RIA test is not accepted as a general diagnostic test because it is not quick and easy to perform, but it is still one of the most sensitive tests for detecting BLV antibodies (51).

A study was done (50) to compare the sensitivity and specificity of AGID and RIA. The sensitivity of AGID compared to gp51 RIA was 85.1% when the test was read at 48 hours and 94.6% at 72 hours. The specificity increased from 92.2% at 48 hours to 96.4% at 72 hours. Reading the AGID at 72 hours also clarified most reactions that were questionable at 48 hours due to haze around the test serum well (50).

Another study (49) was performed to compare the length of time that colostral antibodies were detectable by RIA and AGID in 24 calves. With AGID, average time that colostral antibodies were detectable was 3.8 months (minimum 2 months, maximum 6 months). With RIA, the average time was 6.0 months (minimum 4 months, maximum 9 months). It can be concluded that RIA detects colostral antibodies in older calves later than the AGID test does. This is a disadvantage because it is unclear if a positive test result is due to true infection or colostral antibodies in older calves. That is, there is a longer period of uncertainty with the RIA test.

The ELISA test has been used in the diagnosis of BLV. Portetelle and Mammerickx (53) reported that it is very sensitive and specific and went so far as to say that the ELISA using a monoclonal antibody to bind gp 51 antigen could be the most sensitive and practical method for large-scale detection of BLV. According to Mammerickx and Portetelle, the ELISA can detect BLV in individual cow milk samples (which is not possible with AGID because the antibody level is lower in

milk than in serum). However, in herds with infection rates lower than 5-10%, the ELISA cannot detect antibody in bulk milk tank samples. Like the other serologic tests, the ELISA cannot differentiate colostrally acquired maternal antibodies from active antibodies. Another disadvantage that the ELISA shares with the RIA is that it is so sensitive that it can detect residual colostral antibodies months later than the less sensitive AGID. Contamination of negative sera with a trace of positive serum can be detected by ELISA. There has also been an ELISA developed to detect antibody to glycoprotein 60 (gp 60) antigen, which is an envelope antigen (57).

The Polymerase Chain Reaction (PCR)

The PCR is a procedure for in vitro amplification of a specific segment of DNA (58,59). Within hours, over a million copies of the DNA can be synthesized (59), and this greatly increases the sensitivity of detection of viruses.

Concept of PCR

PCR is a three-step process involving denaturation of double strand DNA to single strands by breaking the hydrogen bonds that hold the 2 strands together, annealing of primers to these separated strands, and synthesis of DNA from the site of primer attachment (60). Heating to 90-100°C is the method used to denature the DNA, though there are other means to do this (61). Synthesis of new strands of DNA is catalyzed by a DNA polymerase that sequentially adds nucleotides complementary to the template strand to the 3' end of the annealed primer (61). After repeated cycles of PCR, the amplified DNA fragment can be identified by gel electrophoresis, among other methods.

Primers

The primers are segments of synthetic single-stranded DNA, usually 20-30 bases in length (oligonucleotides) (60). Two primers are used and each primer is complementary to a 20-30 nucleotide sequence on one of the original DNA strands immediately upstream or downstream to the sequence of interest

(61). The primers hybridize to opposite strands of the target sequence and are oriented so that DNA synthesis proceeds across the region between primers (62). The primers also bind to the extension products allowing DNA synthesis from these as well as from the original DNA, so that each amplification cycle results in doubling of the amount of DNA synthesized in comparison to the previous cycle (62). The result is an exponential accumulation of specific target fragments: approximately 2^n , where n =number of amplification cycles performed (62). A single copy of DNA can be increased up to 1,000,000 copies after only 30 cycles (61). A concentration of between 0.1 and 0.5 μ M of each primer is usually effective (63). At these concentrations, the primers are present in excess so that they are more likely to anneal to the dissociated strands than the strands are to reanneal to each other (61). However, use of higher concentrations of primers increases the possibility of mispriming, accumulation of nonspecific products, and primer-dimers (63). Obviously, nonspecific products use up primers and other reaction components, thereby lowering the yield of the target sequence. The use of higher temperatures for primer annealing and extension increases the specificity of the amplification reaction, which in turn increases the yield of the desired product (64).

There are several guidelines to aid in primer selection (62,63):

1. Primers should have an average G+C (guanosine+cytosine) nucleotide composition of 50-60%.
2. Primers should have a random base composition; that is, if possible, no stretches of polypurines (polyadenosine or polyguanosine) or polypyrimidines (polythymidine or polycytosine).
3. Ensure the primers are not complementary especially at the 3' ends because this promotes primer-dimer artifacts. Primer-dimers are template-independent artifacts that decrease yield of desired product.
4. If possible, palindromic sequences within primers should be avoided because of the potential for a primer to anneal to itself.
5. Significant secondary structure within the template DNA in the area of the target sequence should be avoided.
6. To ensure that the 3' end of the primer anneals strongly to template DNA, primers should be chosen with a 3' C or G (3 hydrogen bonds are more stable than the 2 hydrogen bonds between A and T)(65). The 3' end of the primer is where extension is initiated.

Reaction Conditions

Once the DNA sequence to be amplified and appropriate primers have been selected, the procedure is fairly simple. Theoretically, there is no need to extract the DNA from the

cells because the primers should be able to find the target sequence even in a mixture of DNA, proteins, and lipids (60). The quantity and quality of the DNA to be amplified need not be high: the sample should contain at least one copy of the target sequence, and the concentration of impurities should not be enough to inhibit polymerization (62). Proviral DNA, Taq polymerase, the 4 deoxynucleotide triphosphates, buffer containing magnesium, and the 2 primers are mixed together usually in a microcentrifuge tube. The size of the microcentrifuge tube will be dictated by the size of the sample holes in the heating block of the thermal cycler, if a thermal cycler is used rather than preset water-baths. The usual sample volume is 100 μ l, and typically the sample is overlaid with 100 μ l of mineral oil to prevent evaporation of sample during the cycles of denaturation-annealing-extension.

The four dNTP's or deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP) should be used at the same concentration to minimize misincorporation (62,63). The recommended concentration of dNTP per reaction is between 20 and 200 μ M of each (63). Misincorporation is when the wrong nucleotide is added to the growing DNA chain. For example, dATP may be added to the nascent chain opposite cytosine on the template strand. A-C is not a Watson-Crick pair and dGTP would be the correct nucleotide to pair with the template strand cytosine. Misincorporation is promoted when the concentration of one dNTP is low relative to the others or when the dNTP concentrations are

below 1 μM (63).

The magnesium concentration in the buffer is important because it can affect polymerase enzyme activity, primer annealing, strand dissociation temperatures, product specificity, and primer-dimer formation (63). It is recommended that each PCR reaction contains 0.5 to 2.5 mM magnesium above the total dNTP concentration, because the *Taq* DNA polymerase used in PCR has a requirement for free magnesium above that bound by primers, dNTPs, and template DNA (63).

The standard buffer used for PCRs involving genomic DNA contains 50mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM MgCl_2 , and 100 $\mu\text{g}/\text{ml}$ of gelatin (62). The KCl facilitates primer annealing (63). The gelatin helps stabilize the enzyme, but it is not always essential for the success of the protocol (63). Bovine serum albumin and nonionic detergents (Tween 20 or Laureth 12) can be used instead of gelatin (63).

Taq DNA Polymerase

Taq polymerase is a thermostable DNA polymerase enzyme from the thermophilic bacteria *Thermus aquaticus* (58) that synthesizes DNA by assembling the 4 nucleotides in a 5' to 3' direction (60). The DNA polymerase enzyme previously used was destroyed during the heat denaturation step and fresh enzyme had to be added for each cycle (60). The *Taq* polymerase is a major advance allowing automation of PCR, that is, the use of

thermal cyclers (60). The elevated temperatures at which PCR with *Taq* polymerase can be performed have increased the specificity of priming (65). The optimal concentration for *Taq* polymerase is approximately 2 units/100 μ l reaction (62).

The enzyme has a high temperature requirement (temperature optimum of 80°C), it does not have single-stranded 3'-5' exonuclease activity (at least in vitro), it requires a divalent cation cofactor (Mg^{2+} or Mn^{2+}), and it has high activity in Tris-hydrochloride buffer, pH 7.8 (66). Low levels of monovalent cations (NaCl and KCl) stimulate catalytic activity of the enzyme, but above 100 mM these salts inhibit the activity of *Taq* polymerase (66). The enzyme requires all four dNTP's for good catalytic activity (66). Tindall and Kunkel (67) studied the fidelity of DNA synthesis by *Taq* polymerase. The fidelity of *Thermus aquaticus* DNA polymerase at high temperatures was found to be similar to or better than the fidelity of other exonuclease-deficient DNA polymerases at 37°C (67). *Taq* polymerase is less accurate than the Klenow fragment of *E.coli* DNA polymerase 1, however (67). This can at least partially be explained by the fact that the Klenow polymerase does possess single-stranded 3'-5' exonuclease proofreading activity (67). Using two fidelity assays, it was determined (67) that *Taq* polymerase produces single-base substitution errors at a rate of 1/9,000 and frameshift errors at a rate of 1/41,000. *Taq* polymerase is fast and processive (68): at 70°C the enzyme polymerized at a rate of

approximately 60 nucleotides per second, and, at substrate excess (0.1:1 molar ratio of polymerase to primer/template), most primers were completely extended before extension was started again on new primers.

Taq polymerase was found (68) to have applications in DNA sequencing:

1. It works over a broader temperature range than the other polymerases used.
2. Hairpins in DNA that hinder polymerases and cause termination are not as much of a problem when *Taq* polymerase is used because the high temperatures and low salt concentrations in which the enzyme is active destabilize the hairpins.
3. The enzyme is highly processive, lacks proofreading activity, and has a high turnover number. These features are advantageous in sequencing because they reduce pausing and premature termination at areas with secondary structure.

Thermus aquaticus was first isolated in 1969 (69). It is an aerobic, nonsporulating, nonmotile, gram-negative rod that produces a yellow pigment (69). This thermophilic bacteria has been found in natural aquatic thermal environments (the hot springs in Yellowstone National Park and California), as well as in hot water (69). Brock and Freeze (69) reported that the bacteria's optimal temperature for growth was 70°C (maximum 79°C, minimum 40°C).

Cycling Conditions

As already mentioned, the polymerase chain reaction involves a 3-step process: denaturation, annealing, and extension. This cycle is repeated several times and results in an exponential accumulation of target DNA. Different temperatures are used for each of the 3 steps. Either water baths or a thermal cycler can be used to achieve these temperature changes.

Denaturation of the DNA strands is usually achieved by heating the sample to about 95°C for a short period of time (less than a minute) (62,63). Incomplete denaturation is the most likely cause for failure of PCR (63), so it is important to optimize denaturation temperature and duration. Incomplete denaturation allows the DNA strands to "snap back" (63). This means that the target sequence may be double stranded DNA and not available for primer binding because the primers bind to their complementary sequence on single-stranded DNA. G+C-rich targets may require higher temperatures (63) because it takes more energy to break the 3 hydrogen bonds between G-C pairs than the 2 hydrogen bonds between A-T pairs. Preceding the first cycle with an initial denaturation step of 3 minutes at 93°C is recommended (62).

Annealing is usually performed at around 55°C (range: 40°C to 72°C) (62,63).

Extension is usually performed at 72°C (63). A time of 1 minute for each kilobase of target sequence is a good starting

point (62). The number of cycles depends mainly on the starting concentration of target DNA (63). The more target DNA to start with, the fewer the number of cycles. The use of 30 to 35 cycles is common.

PCR Carry-over

Because of the extreme sensitivity of the polymerase chain reaction, care must be taken to avoid contamination of negative samples with positive samples. This can occur by cross-contamination from the positive control or positive samples during the "before PCR" procedures, or by contamination from already amplified DNA from previous experiments (that is, by performing "before PCR" and "after PCR" procedures in the same location). There are several recommendations of procedures to minimize PCR carry-over (64):

1. The "before PCR" procedures (setting up the reactants in the microcentrifuge tube and addition of mineral oil) should be done in a separate area of the lab or a different room than the "after PCR" procedures (such as the addition of loading dye and loading of the samples into wells in an agarose gel for electrophoresis).
2. Positive displacement pipettes should be used whenever possible, especially when pipetting samples that contain DNA. This type of pipettor does not retain any of the sample.
3. Reagents should be aliquoted; that is, reagents should be stored in small containers rather than large ones. If one vial of a particular reagent becomes contaminated, this

contamination will not affect many subsequent PCRs. To be absolutely safe, a new vial of dNTPs, buffer, primers, and enzyme can be used each time.

4. Careful laboratory technique is essential. Working areas should be cleaned with ethanol often, and periodically depurinated with HCl. Microcentrifuge tube lids should be opened carefully to prevent aerosol cross-contamination.

5. Positive, negative, and reagent controls should be used. A positive control is known to contain the DNA sequence of interest. A negative control contains non-target DNA. A reagent control contains all reagents (dNTPs, enzyme, primers, and buffer), but no DNA.

Uses of PCR

The polymerase chain reaction is a fairly recent advance in molecular biology. Since 1986 references to methodology and uses of PCR have increased considerably. It has been used in the laboratory to facilitate research, and it is currently used extensively in human medicine. Its potential use in veterinary medicine is only recently being realised.

Molecular Biology Research

The polymerase chain reaction has greatly facilitated research in molecular biology, in both pure science and in science that has applications in medicine and forensics.

Innis et al. (68) report that the polymerase chain reaction using *Taq* polymerase is highly suited for DNA sequencing.

Steps in this procedure are as follows (68):

1. A primer is annealed to a single-stranded DNA template (this can be denatured double-strand DNA or DNA that is single-stranded).
2. The primer is extended with DNA polymerase enzyme in 4 separate reactions. Each reaction mixture contains one *alpha*-labeled dNTP, a mixture of unlabeled dNTPs, and one chain-terminating ddNTP.
3. The product of each reaction is resolved on a polyacrylamide/urea gel .
4. An autoradiograph is taken of each gel, and the image is examined to determine the sequence.

Improvements in the speed and automation of this sequencing technique were made using the PCR to selectively amplify DNA. This is particularly suited for segments of single-copy genomic DNA (68). There is no need to add enzyme after each cycle when using thermostable *Taq* polymerase, and it can be used at high annealing and extension temperatures which increases the yield, specificity, and length of products that can be amplified (68).

DNA sequencing of human genes is used to identify mutations and polymorphisms (70). Traditionally, if an individual is suspected of having a mutation, his or her genes are cloned, then compared to the sequence of cloned DNA from the normal allele (70). Also, if the person is a heterozygote rather than a homozygote, then the process becomes more complicated

because both alleles have to be detected (70). If the exact nature of the mutation is known, then hybridization with mutation-specific probes can be used for detection (70). However, these probes, known as allele-specific oligonucleotides (ASO), will only anneal to DNA sequences that match them perfectly (71). There are other approaches (70) that do not provide the actual sequence at the mutation site. Direct genomic sequencing avoids the need for lengthy cloning procedures and provides the actual DNA sequence of the mutation, but it is difficult to do because the human genome is complex (70). Use of the PCR to amplify the region of interest reduces this problem, and was used (70) to amplify and sequence parts of the human *B*-globin and fetal globin genes. The use of PCR in sequencing further simplifies the procedure because DNA purification is not required for PCR amplification: it can be done directly on crude cell lysates (71). Scharf et al. (72) used the PCR to facilitate cloning: by amplifying parts of the human genome by PCR and directly cloning into an M13 vector, the need for extensive genomic library construction, screening, mapping, subcloning, and sequencing was reduced.

A modification of the polymerase chain reaction has been used to create point mutations into human *ras* oncogenes and to amplify these mutated copies (73). The PCR is used to detect point mutations in genes, but there is usually no positive control DNA that contains the mutation (73). To address this

problem in the *ras* oncogene, Rochlitz et al. (73) developed a technique using single base mutations in codons 12 and 61 of this gene. A normal 5' primer was used for the PCR, but the 3' primer contained a single base mismatch to the target codon. The 3' primer also acted as the hybridization probe when screening for the mutation.

The c-Kirsten *ras* oncogene is present in the human breast carcinoma cell line MDA-MB231 (74). Using the polymerase chain reaction to amplify parts of the human c-Kirsten *ras* gene and hybridization of this amplified DNA to oligomers specific for a mutation in codon 13 of the gene, researchers (74) showed that this mutation, which results in a change from glycine to aspartic acid in the c-Kirsten *ras* amino acid sequence, activates the oncogene.

The PCR has been used to amplify DNA that has been inserted into a plasmid vector (75). It is believed that any insert cloned in a vector can be amplified if there are available flanking primers and if the insert is not over 2 kb in length (75).

Conventional PCR amplifies the region of DNA between the primers. An interesting technique known as "inverse PCR" is an extension of the polymerase chain reaction such that regions that are outside or next to an area of known nucleotide sequence can be amplified (76). The inverse PCR can be summarized as follows (76):

1. The region to be amplified is the upstream and downstream

regions of DNA to a core region of known sequence.

2. The DNA (genomic DNA, for instance) is cut using a restriction enzyme that does not have restriction sites within the core region.

3. The DNA fragments produced by the restriction enzyme digestion are ligated with a ligation enzyme such as T4 DNA ligase to produce circularized DNA.

4. The circularized DNA is then subjected to PCR. The primers are complementary to the ends of the core sequence, but are oriented such that the polymerase enzyme synthesizes DNA into the region of unknown sequence.

5. The major amplification product is a linear double-stranded segment of DNA that contains regions 3' and 5' to the core sequence.

The inverse PCR allows rapid amplification of unknown sequences of DNA, but it is limited by the size of a region that can be amplified by PCR (2-3 kb) (76).

The use of PCR coupled with sequence-specific oligonucleotide (SSO) probe hybridization was found to be effective in analysis of partially degraded DNA extracted from formalin-fixed, paraffin-embedded tissue specimens that had been stored for several years (77). The tissues were from patients with abnormal and normal B-globin genes, and the DNA that was extracted was degraded in all the samples, as evidenced by small segments of DNA seen on a 1.5% agarose gel (range: 0.1-24 kb) (77). Despite the degradation, 5'-end-

labelled oligonucleotide probes for normal, sickle cell, and hemoglobin C β -globin alleles bound correctly to the DNA extracted from the histologic specimens (77). A similar procedure was performed on 40 year-old, paraffin-embedded tissues from patients with cervical carcinoma or squamous cell carcinoma of the cervix (78). Most molecular techniques require high-molecular-weight DNA, so the PCR has an advantage in that it works on small DNA segments (77,78). There is, therefore, great potential to use archival formalin-fixed, paraffin-embedded tissues in studying diseases at the DNA level over time, without having to perform prospective studies which can take years (77,78).

The polymerase chain reaction has been used to amplify small segments of mitochondrial DNA from a 7,000-year-old human brain (79). The cranium containing the brain was found in a flooded sinkhole in Florida in 1986 (79). DNA was extracted and purified from neural tissue, and then PCR was performed on it. Bovine serum albumin (2 μ g/ml) and a high concentration of *Taq* DNA polymerase were necessary to overcome an unknown factor in the brain that inhibited amplification (79). Most of the DNA from the brain had degraded to a small molecular size (50-200 bp), which is typical of ancient DNA (79). The researchers (79) have noted a difference in amplification efficiency of ancient DNA versus modern DNA: in ancient DNA there is an inverse relationship between the efficiency of amplification and the length of the sequence to

be amplified. Segments of ancient DNA up to 120 bp could be amplified, but segments of 200 bp could not be amplified; whereas with modern human DNA up to 2 kb could be amplified (79).

DNA typing has been done from single hairs (80), and this has great potential in forensic and medical use. Most of the DNA in a hair is in the root and surrounding sheath cells, so that shed hairs contain less DNA (up to 10ng) than freshly plucked hairs (up to 200ng) (80). At the site of a crime the hairs are usually shed hairs from several people and there is usually only one or a few hairs from any one person, so that DNA typing of single, shed hairs is more useful than typing of pooled, freshly plucked samples (80). By the use of the PCR to amplify DNA (80), DNA sequences from the root of shed and freshly plucked single hairs could be detected. Collection of hair samples rather than blood may be useful when transport of blood may be difficult or in endangered or dangerous animals (80).

Another interesting application of the PCR is in amplification of DNA in genetically engineered microorganisms (81). There has been interest in introducing genetically engineered bacteria into the environment, and in detecting these bacteria in environmental samples (81). However, there have been difficulties because the methods that have been used lack sensitivity. Steffan and Atlas (81) used the polymerase chain reaction to amplify a small specific region of DNA from

the herbicide-degrading bacterium *Pseudomonas cepacia* after it had been introduced at several different concentrations into environmental samples with mixed bacterial populations. Using the PCR, *P. cepacia* was detected at a concentration of 1 cell per gram of sediment, which is a 1,000-fold increase in sensitivity over nonamplified samples (81).

Human Medicine

In human medicine the PCR has greatly facilitated the diagnosis and monitoring of many disease processes, especially those with a viral etiology and those involving defects in the genome. Serology or culture may be difficult in diseases caused by viruses, in which case the PCR may be very useful (61). The PCR may also be especially valuable in cases where early detection of infection is important (61).

Recent developments in molecular biology including PCR have enhanced detection of mutations that may cause genetic disorders in man and animals (82). The PCR is used in the diagnosis of chronic myeloid leukemia and in following the course of treatment (83): it is used to amplify a sequence that includes an area of gene translocation (the bcr-abl translocation) found in patients with this disease. Patients with certain types of muscular dystrophy have a defective gene encoding the muscle protein dystrophin (84). Dystrophin mRNA is not easily studied because it is a large molecule and makes up only 0.01-0.001% of total muscle mRNA (84). The PCR was used to amplify dystrophin mRNA to facilitate study of the

dystrophin gene transcript (84). To perform PCR on mRNA, a DNA copy of the mRNA is made using a reverse transcriptase enzyme and the PCR subsequently performed on the cDNA (84). Alpha-1-antitrypsin deficiency can be diagnosed prenatally using the polymerase chain reaction (85). The polymerase chain reaction is one of the tools of potential use in the diagnosis of chronic active and recurrent herpesvirus infections (86). With the assistance of DNA amplification by PCR, sufficient DNA can be isolated from buccal epithelial cells obtained by mouthwash for direct gene analysis (87). The authors believe this to have potential use in screening for carriers of single gene defects such as cystic fibrosis (87).

Patients with *B*-thalassaemia have a mutation(s) in the *Beta*-globin gene resulting in decreased synthesis of the *B*-globin protein. In order to characterize the mutation(s) in the single copy *Beta*-globin gene in 5 patients with *B*-thalassaemia, PCR was used to amplify the genome for sequence analysis (88). Two previously uncharacterized mutations in the *Beta*-globin gene were found by this method (88).

Enterotoxins of 2 bacteria (enterotoxigenic *Escherichia coli* and *Shigella*) cause a significant amount of acute diarrhea in children in Third World countries (89). The polymerase chain reaction was used to amplify the genes encoding the heat-stable (ST) and heat-labile (LT) enterotoxins of enterotoxigenic *Escherichia coli* (ETEC) and the invasion-associated loci (*ial*) of the large *Shigella*

virulence plasmid (89). The procedure was then tested in a community in southeastern Mexico, and the authors reported that it was relatively rapid, straightforward, inexpensive, and was more sensitive than other diagnostic tests (89).

The PCR also has applications in diagnostic pathology (90). For instance, PCR amplification of DNA from extremely small samples would be very helpful in forensic science (90).

Bangham et al. (91) were interested in identifying the retrovirus associated with TSP (tropical spastic paraparesis), a chronic neurologic disease. Serologically, most TSP patients are positive for HTLV-1 (human T-cell lymphotropic virus type 1). The authors used the PCR to amplify part of the genome of 5 TSP patients, and sequenced part of the polymerase gene. They showed that the virus associated with TSP is indeed HTLV-1, and that patients may have more than one variant of the virus (91).

HIV-1 (human immunodeficiency virus-1) is the causative agent of AIDS (92). Infection with HIV-2 is less characterized and has been reported in much fewer people than infection with HIV-1 (92). A study was performed to find the prevalence of HIV-1 and HIV-2 infection in a region of West Africa (92). The authors used several serologic tests, as well as PCR amplification of proviral DNA. This region in West Africa is endemic for both HIV-1 and HIV-2, yet most infections were either exclusively HIV-1 or HIV-2. There were two patients with AIDS who were only infected with HIV-2, and one patient

was dually infected with HIV-1 and HIV-2 (92).

Several types of PCR assays have been designed that could assist in investigations of neoplasia at the molecular level and may have use in cancer diagnosis (93). A major problem in treatment for cancer is recurrence of disease, probably because residual neoplastic cells are clinically undetectable (94). When the concentration of neoplastic cells is below 1% the cancer is very difficult to detect, yet a concentration of cells this low theoretically occurs in cancer patients in remission (94). PCR was used to amplify the area in the genome that encompasses the crossover sites of a chromosomal translocation for follicular lymphoma, t(14;18) (94). This technique proved to be very sensitive in detecting residual neoplastic cells carrying t(14;18), more sensitive than Southern blot analysis, and the authors believed the technique to have potential in identification of subclinical cancer (94).

Veterinary Medicine

The potential uses of the PCR in veterinary medicine are tremendous. Deacon and Lah (95) believe that the PCR has both research and diagnostic applications in veterinary medicine. For instance, "inverted" PCR can be used to amplify regions outside the primers. This has been used to analyze integration sites of the type C retrovirus gibbon ape leukemia virus in a certain tumor cell line (95). The need for only a small specimen for PCR can be advantageous in the diagnostic lab

(95). Because PCR is so sensitive, it may lead to earlier detection of bacterial and viral disease (95). PCR can be used to detect genetic disorders in animals, and can be used in DNA fingerprinting to identify individual valuable animals (95). PCR also has the potential to expedite genetic improvement programs in animals (95).

The polymerase chain reaction has been used to amplify DNA sequences of pseudorabies virus (porcine herpesvirus 1) from infected cell cultures as well as cells of acutely infected pigs and latent carriers (96). Porcine herpesvirus 1 causes high mortality in piglets, but adult pigs usually have a latent infection and periodically shed the virus, causing recurrent infections in fetuses and piglets (96). Therefore, it is important to detect latent infection as well as acute infection. PCR amplification of the viral DNA coupled with detection with an oligonucleotide probe proved to be a very specific and sensitive method of diagnosing infection by pseudorabies virus (96).

Hemophilia B is a bleeding disorder caused by absence or malfunction of a circulating blood coagulation glycoprotein known as factor IX (97). The treatment for this disorder is infusion of plasma rich in factor IX during each episode of bleeding (97). Repeated blood infusions increase the risk of inadvertant introduction of infectious agents into the patient's bloodstream (97). Gene therapy has been proposed as a treatment for hemophilia B, and since dogs get hemophilia B,

canine hemophilia B could be used as a model for human hemophilia B (97). The canine factor IX gene was amplified by PCR and a single missense mutation in the hemophiliac sequence was found (97). It was theorized that the mutation has effects on the tertiary structure of the factor IX molecule. This discovery has potential to facilitate research into gene therapy of human hemophilia B (97).

Bluetongue virus (BTV) is an arthropod-borne Reovirus which infects cattle and sheep, though clinical disease is predominantly seen in sheep (98). The viral genome is a segmented double stranded RNA (98). Gould et al.(98) used the polymerase chain reaction in the detection of BTV in the blood of an infected sheep. Use of the PCR increased the speed and efficiency of identification of bluetongue infection because there was no need to amplify the sample by lengthy procedures such as egg passage or tissue culture (98).

MATERIALS AND METHODS

Source of Cattle and Samples
Collected

Cattle used in this study were adult Holstein dairy cows belonging to Utah State University, Logan, Utah. The animals were housed at Caine Dairy, Wellsville, Utah. This herd has been screened for enzootic bovine leukosis by AGID since 1985 and has overall percent positive BLV titers ranging from 21.9 in 1985 to approximately 40. Whole blood samples were collected from 3 cows positive for BLV by the AGID test and the PCR test. These cows were identified as 104, 6372, and 6490. The 3 samples were mixed and the composite blood used to infect 10 cows that were negative for BLV by AGID in the 1989 herd check. Five of the cows (278, 5720, 6618, 6756, and 6820) were inoculated with 0.1 ml of blood, and the other cows (6446, 6508, 6516, 6546, and 6696) were inoculated with 1 ml of blood. The animals were infected by intramuscular injection. A minute amount of virus-infected blood was considered sufficient to transmit the disease since the virus can be transmitted by insects (28,29,30,31), which carry minute amounts of blood. Experimentally (34), 2 groups of calves were given 10 microliters and 1 microliter of whole blood from an infected animal and seroconverted within 8 weeks and 14 weeks, respectively, after inoculation. The route of administration (intramuscular, intravenous, subcutaneous, or intradermal) did not affect the rate of seroconversion.

Immediately before the 10 experimental cows were infected, blood samples from each were collected for the AGID test and for lymphocyte isolation to check if the cows were negative for BLV by AGID and PCR before being inoculated. Animals were bled from the tail vein into red top (no anticoagulant) vacutainer tubes for serum collection and into lavender top (EDTA) tubes for DNA extraction. Approximately 10 ml of blood was collected in each tube.

The 10 cows were then bled on day 1 (day 0 being day of inoculation) and day 7 post-inoculation, and then every 2 weeks subsequently until 3 months post-infection. At each collection period, blood for the AGID test and for PCR was collected from each cow.

Initial Sample Treatment

The coagulated blood was centrifuged at 1500 rpm for 10 minutes, and the serum was decanted and frozen. The EDTA-treated blood was layered over Histopaque-1077 (Sigma Diagnostics, St. Louis, Missouri), centrifuged at 1500 rpm for 30 minutes, and the mononuclear cell layer pipetted into clean tubes. The lymphocytes were washed twice with 5 ml of phosphate buffered saline (PBS). Finally, 465 microliters of digestion buffer was added to each sample before they were frozen at -80°C . This ficoll gradient procedure was done in duplicate so that there were 2 of each sample of lymphocytes.

The Agar Gel Immunodiffusion Test

The AGID tests were all performed at 1 time at the end of the experiment to minimize variation due to reagents and experimental conditions. The serum samples were allowed to defrost and the Leukassay B test kit (Pitman Moore, Mundelein, Illinois) was used to test for antibodies to BLV. The test was run 3 times on each serum sample, and the samples run in a different order each time. Test serum, weak positive control serum, positive control serum, negative control serum, and antigen were loaded into wells in the agar. The plates were incubated in a humidified chamber for a total of 72 hours. The plates were read at 48 hours and 72 hours. If antibody was present, it formed a line of precipitation with the antigen.

DNA Extraction

The lymphocytes (with digestion buffer) were defrosted, 35 μ l of proteinase K (10 mg/ml) added, and the samples digested in a 55°C waterbath overnight to remove most of the protein. Additional purification of the DNA by phenol/chloroform extraction was then performed. The DNA solutions were extracted with phenol, then with a 1:1 phenol and chloroform mixture, and finally with chloroform. Then 0.1 volume 0.5M sodium chloride was added to each sample. The DNA was precipitated with 100% ethanol, washed with 70% ethanol, and suspended in 50 μ l TE buffer, pH 8.0. The samples were then ready for amplification.

Primers Used

The primers were from the *gag* region of the BLV genome within the area coding for the p24 protein. Both primers were 20 nucleotides in length. The nucleotide sequence that was used to select the primers was from *RNA Viruses* (99). Primer 1, complementary to the negative sense DNA strand, started at position 758 in the genome and had the following sequence: 5'-AAGGAAATCGCAACCGCCAT-3'. Primer 2, complementary to the positive sense strand had the following sequence: 5'-CTGAAGGACGAGTAGGGAGA-3'. This sequence is complementary to the 20 nucleotide sequence of the BLV genome that ends at position 1133. Therefore, the region that was amplified was 375 bases in length (1133-758=375). Primers were synthesized by Dr. Tom Grover at the Utah State University Biotechnology Center, Logan, Utah.

The Polymerase Chain Reaction

The 3-step cycle for the polymerase chain reaction involves denaturation, annealing, and extension. Thermostable DNA polymerase from *Thermus aquaticus* (Promega Corporation, Madison, Wisconsin) was used for extension. The cycling was done automatically in a thermal cycler (Perkin-Elmer Cetus, Emeryville, California). The program used was as follows:

1. Time delay: 3 minutes to get up to 95°C.
2. 30 cycles of denaturation, annealing, and extension:
 - A. Denaturation: 1 minute to get up to 95°C.

30 seconds at 95°C.

B. Primer annealing: 1 minute to get down to 55°C.

30 seconds at 55°C.

C. Primer extension: 30 seconds to get up to 72°C.

1 minute 30 seconds at 72°C.

3. Time delay: 30 minutes at 72°C. This is to ensure that extension is complete and that the amplified DNA is double-stranded (62).

4. Soak file: 25°C until machine turned off.

A total volume of 100 ul per PCR reaction was as follows: 49.5 ul double-distilled water, 10 ul (0.25 uM) each primer, 10 ul dNTPs (from a stock solution containing 2 mM each dNTP), 10 ul PCR buffer (*Taq* DNA polymerase 10X buffer: 500 mM KCl, 100 mM Tris-HCl (pH 8.8 at 25°C), 15 mM MgCl₂, 1% Triton X-100), 0.5 ul *Taq* polymerase (2.5 units), and 10 ul sample DNA. The PCR mixture was overlaid with 100 ul mineral oil. For the reagent control, a volume of 59.5 ul of double-distilled water was added instead of DNA solution to make a total volume of 100 ul.

To increase the yield of PCR product so that it would be detectable by ethidium bromide gel electrophoresis, a second PCR was performed on each sample. After the first PCR this was accomplished by adding 10 ul of each primer, 0.5 ul of *Taq* polymerase, 10 ul of dNTPs, and 9.5 ul of double-distilled water to each sample (total added volume of 40 ul). The samples were then put back in the thermal cycler for another

30 cycles of denaturation, annealing, and extension.

Positive and Negative Control DNA

The positive control DNA used in the PCR was extracted from FLK-BLV cells (fetal lamb kidney cells infected with BLV). The FLK-BLV cells were a gift of Dr. M.J. Van der Maaten. The negative control DNA was extracted from MDBK cells (a line of bovine kidney cells known to be negative for BLV). The MDBK cells were provided by Yoko Elsner in the Animal, Dairy, and Veterinary Science Department at Utah State University, Logan, Utah. Dr. Gregoire Marsolais extracted and froze the DNA from these cells.

Agarose Gel Electrophoresis

After PCR, gel-loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol in water (100)) was added to each sample, and the samples were loaded onto a 1.5% agarose gel stained with ethidium bromide at a concentration of 0.5ug/ml electrophoresis buffer. Agarose gel electrophoresis is a method for separating and purifying fragments of DNA (100). A 1.5% agarose gel effectively separates linear DNA fragments ranging in size from 3 to 0.2 kb (100). The anticipated PCR product, 355 bp (0.355 kb), is within this range. The gel was attached to a power supply and an electric field generated. The gel was run at 40 volts for 2-4 hours then photographed on a UV transilluminator. One Kb DNA ladder standard size marker (Bethesda Research Laboratories) was run on the gel

simultaneously as reference DNA of different sizes. Test samples and positive, negative, and reagent controls were run on the same gel.

Southern Blot and Hybridization

To ensure that the DNA band observed on the agarose gel was the amplified 355 bp sequence in the gag region of the BLV genome, the DNA band was transferred to a membrane filter (Gene Screen, Dupont, New England Nuclear) and hybridized with a probe (plasmid pUX-Gag, generous gift of David Derse) containing the long terminal repeat and all of the gag gene of BLV. This procedure was only performed on 1 gel that had kb ladder, reagent control, negative control, positive control, and 5 samples were run on it. Once the band had been identified as the correct band, the Southern blot and hybridization was not run for every gel.

The Southern blot was done using a pressure blotting system (Posiblot Pressure Blotter & Pressure Control Station, Stratagene, La Jolla, California). Prior to Southern transfer, the gels were pre-treated according to the manufacturer's recommendations as follows:

1. Depurination: The gel was submerged in 0.25 N HCl and gently shaken for 30 minutes.
2. Denaturation: The HCl was poured off and 0.5 N NaOH, 1.5 M NaCl solution poured over the gel. The gel was submerged in the NaOH, NaCl solution for an hour.
3. Neutralization: The denaturing solution was poured off and

replaced with 1 M Tris pH 7.5, 1.5 M NaCl solution. This was poured off after an hour.

The blotter was assembled and the blot performed according to the manufacturer's instructions. After the Southern blot, the "Gene screen" membrane onto which the DNA had been transferred was dried in an 80°C oven for 2 hours.

The nucleic acid probe was labeled with alpha-32P deoxyCTP (New England Nuclear) with a Random Primer Reaction kit (Boehringer Mannheim).

The hybridization procedure was carried out as follows (100): The membrane was placed in a sealable bag and prehybridized overnight at 42°C in 10 ml of a solution containing 5X SSC, 5X Denhardt's solution, 25 mM KPO₄, pH 7.4, 50 ug/ml salmon sperm DNA, and 50% formamide. The hybridization solution consisted of the prehybridization solution with 10% dextran sulfate. The probe was used at a level of 500,000 cpm per ml of hybridization solution. The prehybridization solution was removed from the bag and replaced with 10 ml of hybridization solution. The membrane was hybridized overnight at 42°C, then it was washed twice for 15 minutes in 1X SSC, 0.1% SDS at room temperature, and it was then washed twice for 15 minutes in 0.25X SSC, 0.1% SDS at hybridization temperature. Finally, the membrane was exposed to X-ray film.

The PCR Test with BIV

Bovine leukemia virus is a retrovirus. It, like all tumor-producing retroviruses, is in the subfamily *Oncovirinae* (20). Another subfamily of the retroviruses is the *Lentivirinae* (20), which are nononcogenic viruses causing chronic diseases (101). Bovine immunodeficiency-like virus (BIV) is a recently characterized bovine lentivirus (101). Because BIV infection is believed to be fairly widespread (101), and because it is a retrovirus, BIV-infected tracheal epithelial cells (a gift of Matthew Gonda) were processed for PCR using the BLV primers to make sure that infection with BIV would not give false positive results to the PCR test for BLV. The procedure performed on BIV was identical to that performed on BLV.

RESULTS

The purpose of this study was to develop a diagnostic test for BLV using the PCR, then to compare the PCR test with the AGID test, which is currently used extensively in the diagnosis of BLV.

Five of the experimental cows (278, 5720, 6618, 6756, and 6446) were negative for BLV by the PCR and the AGID immediately before they were infected. Table 1 shows the AGID results for these cows since 1985, which is when yearly testing for BLV was started in the Utah State University dairy herd. The other 5 cows were positive for BLV by the PCR and the AGID just before inoculation and remained positive throughout the experiment.

The AGID tests were read at 48 and 72 hours. The results at 72 hours were no different than the results at 48 hours. The test was run 3 times and the results of these 3 trials were very consistent. The only differences were that some weak positive results in some trials were strongly positive in others. Figure 1 is an AGID plate showing a positive reaction, a weakly positive reaction, and a negative reaction. Table 2 shows the samples that gave a weakly positive reaction in some trials and a strongly positive reaction in others.

TABLE 1-AGID test results of 5 experimental cows since 1985

YEAR	1985	1986	1987	1988	1989	1990
<hr/>						
COW #						
278	*	*	NEG	NEG	NEG	NEG
5720	NEG	NEG	NEG	NEG	NEG	NEG
6446	*	*	NEG	NEG	NEG	NEG
6618	*	*	NEG	NEG	NEG	NEG
6756	*	*	*	NEG	NEG	NEG

* Cow was either not in herd or not yet born.

NEG=Negative AGID test result

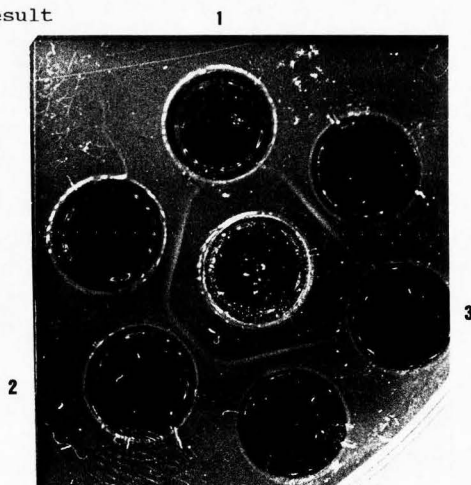


Fig 1-Agar gel immunodiffusion test. Well 1 = weak positive, well 2 = positive, well 3 = negative.

TABLE 2-AGID test results that were weakly positive in one or two trials and strongly positive in the other trial(s)

	TRIAL 1	TRIAL 2	TRIAL 3
WEAK POSITIVES	NONE	5720 on 12/1 6820 on 11/3 6618 on 10/7 6446 on 10/7	5720 on 12/1

With the AGID test, 3 cows (6446, 6618, 6756) had become positive by 9/22/90 (inoculation date for all cows was 9/1/90). Therefore, these 3 cows seroconverted between 1 and 3 weeks post-inoculation. The other 2 cows (278, 5720) had seroconverted by 5 weeks post-inoculation (positive for the first time on 10/7/90). These 2 cows, therefore, seroconverted between 3 and 5 weeks post-inoculation. All cows remained positive for the remainder of the study (last testing date was 12/1/90) after they had initially seroconverted. Table 3 summarizes these results. With the PCR test, samples from all 5 cows were positive by 9/8/90, which is only 7 days post-infection (table 4). Therefore, the PCR detected infection with BLV between day 2 and day 7 post-inoculation.

TABLE 3-AGID results at each sample collection period

COW	DAYS POST-INOCULATION								
	0	1	7	21	35	49	63	77	91
278	NEG	NEG	NEG	NEG	POS	POS	POS	POS	POS
5720	NEG	NEG	NEG	NEG	POS	POS	POS	POS	POS
6446	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS
6618	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS
6756	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS

NEG=Negative AGID test result POS=Positive AGID test result

DAY 0=Day of inoculation

Figure 2 is the agarose gel that was blotted and hybridized. The positive control band is clearly seen, followed by 3 positive samples and 2 negative samples. The x-ray film of the hybridization of this gel (figure 2B) shows the positive control band and the 3 positive samples. The results of the hybridization show that the bands of DNA that were observed on the agarose gel were, indeed, the target sequence in the gag region of the BLV genome.

Figure 3 is an example of 2 agarose gels after electrophoresis and staining with ethidium bromide. The DNA samples in figure 3A were from samples taken immediately before experimental inoculation. Five of the cows were negative for BLV by PCR and the other 5 cows were positive on

TABLE 4-PCR results at each sample collection period

COW	DAYS POST-INOCULATION								
	0	1	7	21	35	49	63	77	91
278	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
5720	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
6446	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
6618	NEG	NEG	POS	POS	POS	POS	POS	POS	POS
6756	NEG	NEG	POS	POS	POS	POS	POS	POS	POS

NEG=Negative PCR test result POS=Positive PCR test result

DAY 0=Day of inoculation

day zero. Figure 3B is an agarose gel of DNA from cows taken on day 7 post-inoculation. Two of the samples on the gel are from cattle positive by PCR but negative by AGID on day 7. The fact that all bands observed were at the same position on the gel, and the fact that these bands corresponded approximately to a band of 344 base pairs on the DNA size standard strongly suggest that the amplified DNA that was the 375 base pair target sequence in the *gag* region of the genome.

The PCR performed on BIV gave negative results. In other words, the test using primers specific for BLV did not give a false positive result.

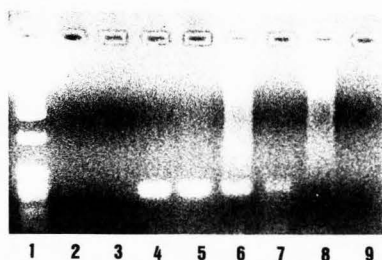


Fig 2-Agarose gel of PCR-amplified DNA and southern blot of this gel. Fig 2A-Agarose gel of PCR-amplified leukocyte DNA. · = position of 344 base pair molecular weight standard. Lane 1 = molecular weight standards, lane 2 = reagent control, lane 3 = negative control DNA, lane 4 = positive control DNA, lanes 5,6,7 = BLV positive cattle, lanes 8,9 = BLV negative cattle.

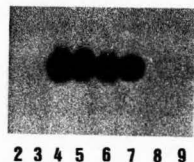


Fig 2B-Southern blot of gel in Fig 2A.

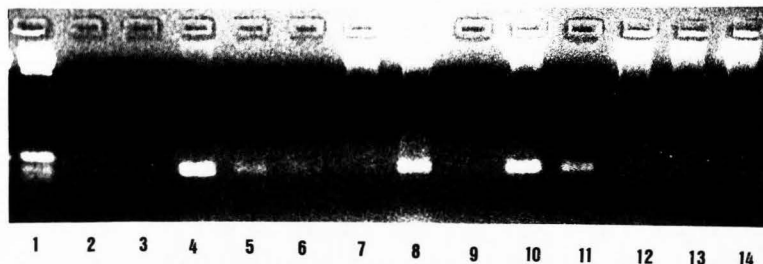


Fig 3-Agar gel electrophoresis of PCR-amplified leukocyte DNA and control samples from experimentally infected cattle. Fig 3A-Samples taken on day 0 before inoculation.

• = position of 344 base pair molecular weight standard. Lane 1 = molecular weight standards, lane 2 = reagent control, lane 3 = negative control DNA, lane 4 = positive control DNA, lane 5 = cow # 6508*, lane 6 = cow # 6546*, lane 7 = cow # 6618\$, lane 8 = cow # 6696*, lane 9 = cow # 6756\$, lane 10 = cow # 6820*, lane 11 = cow # 6516*, lane 12 = cow # 6446\$, lane 13 = cow # 5720\$, lane 14 = cow # 278\$. * = positive by both AGID and PCR before inoculation, \$ = negative by both AGID and PCR before inoculation.

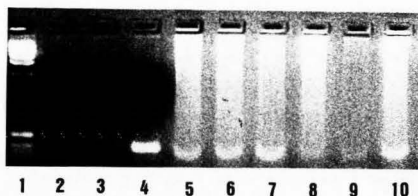


Fig 3B = PCR amplification of leukocyte DNA at day 7.

· = position of 344 base pair molecular weight standard. Lane 1 = molecular weight standards, lane 2 = reagent control, lane 3 = negative control DNA, lane 4 = positive control DNA, lane 5 = cow # 6446@, lane 6 = cow # 6508, lane 7 = cow # 6516, lane 8 = cow # 6546, lane 9 = cow # 6618@, lane 10 = cow # 6696. @ designates cattle positive by PCR but negative by AGID on day 7.

DISCUSSION

This experiment was designed as a pilot study to develop a PCR protocol that would be specific for BLV, to compare the sensitivity and specificity of this test to the AGID test, and to assess the feasibility of widespread use of the PCR in the diagnosis of BLV.

Because 5 out of the 10 cows used in the study were positive for BLV before inoculation, only 5 cows were infected with BLV as a result of experimental inoculation. Though experimental animal numbers were small, the results do answer the hypothesis that the PCR is more sensitive and specific than AGID.

There are many variables to be addressed in the initial development of the PCR protocol. First and foremost is the selection of effective primers. Guidelines for primer selection have been given elsewhere (literature review section in this paper). Though the primers used in this study are not ideal in every aspect, they do have an average G+C content of 50-60%, they are not complementary to each other, they are not palindromic, and there are no stretches of polypurines and polypyrimidines within the primers that are longer than 3 base pairs. Guidelines have been given for the concentration of all the components in the PCR and the cycling conditions. In most circumstances, these conditions will be effective when amplifying genomic DNA. However, the PCR conditions should be optimized. In this experiment, the primers selected the first

time were effective, but the PCR conditions needed to be optimized. Functional conditions were determined before the test was run on the stored experimental samples.

A PCR protocol was successfully developed for bovine leukemia virus. Another bovine retrovirus, BIV, was found not to give a false positive result to the PCR test developed for BLV. Ideally, the PCR test for BLV should be performed on all common bovine viruses to ensure that infection with BLV is the only way that an animal would be positive to the test.

An important consideration when developing a PCR protocol is the geographic location where the test will be used. The primers successfully used in Logan, Utah were not consistently successful in Quebec (102) so other primers had to be selected. Whether this is an optimization problem of the PCR reaction, or whether it reflects regional variations in the BLV genome is not clear. To address this question, BLV isolates from Quebec and Logan would have to be sequenced and the nucleotide sequences compared.

The 5 cows that were positive before the experimental inoculation were kept in the study. They remained positive for BLV by AGID and PCR throughout the experiment. These positive samples were tested by AGID and PCR concurrently with negative samples at the beginning of the study before all the cows became positive for BLV. This suggests that there was no cross-contamination between samples in the AGID and PCR tests. Therefore, the 5 positive cows may have served as quality

control, at least in the early stages of the experiment, though this was not the original intent when the cows were included in the study. Initially, 10 cows that were negative for AGID in the 1989 herd check and that were destined for slaughter were chosen to be on the study. Five of the cows seroconverted after the 1989 herd test for BLV, so that only 5 cows were negative for BLV at the start of the study.

The results of this study indicate that the use of PCR provides diagnosis of BLV sooner after infection than the AGID test.

In this experiment, cows became AGID-positive between 1 and 5 weeks post-infection. This is similar to previous work (23) in which animals first became AGID-positive at 5-6 weeks after inoculation.

Naif et al (103) recently used the PCR to detect BLV proviral DNA in dairy and beef animals, some of which had persistent lymphocytosis and some others had lymphosarcoma. They used primers from the *env* gene in an Australian isolate of BLV and amplified a DNA fragment 440 bp in size (103). These researchers (103) detected BLV by PCR in animals that were AGID-positive, AGID-positive with persistent lymphocytosis, and AGID-positive with tumors. They found that PCR was negative in AGID-negative animals, except in 1 case. Naif et al (103) concluded that the PCR is a sensitive, fairly rapid, and inexpensive test. In another study (104), these researchers used the PCR to detect BLV infection in sheep. The

sheep were infected by intravenous injection of lymphocytes from either a cow or a sheep known to be BLV positive (104). In one phase of the experiment (104), 4 out of 6 sheep became BLV positive by the PCR 1 week after infection, and the other 2 animals had become BLV positive at 3 weeks post-infection.

In this study, all 5 cows that were infected became positive by the PCR test 7 days post-innoculation. This is comparable to the study performed in sheep (104), in which 4 out of 6 sheep became positive by PCR 1 week post-infection. It can, therefore, be concluded that PCR is a more sensitive test than the AGID and can detect infection at least 2 weeks before the animal seroconverts. Naif et al (103) found that 1 AGID-negative animal was, in fact, BLV-positive by the PCR. This suggests that PCR is more specific than AGID; that is, there is more chance of false negatives with the AGID test.

Despite the fact that the PCR is highly sensitive and specific, relatively quick and easy to perform once the protocol has been optimized, relatively inexpensive unless a thermal cycler has to be purchased (and this would be a one-time cost of \$3,000 at the very least), theoretically able to distinguish an AGID-positive test result due to colostral antibody from that acquired by natural infection (because it detects the actual viral DNA), and theoretically able to detect virus in milk, it may not be accepted into widespread use. There are a number of reasons for this:

1. The AGID test is rapid, easy to perform, inexpensive, and

is widely used and accepted. There is little need for a new diagnostic test, at least for herd screening.

2. PCR is relatively new technology, and few veterinary diagnostic labs have the equipment and expertise to perform it.

The PCR will have its greatest application in testing individual animals for specific reasons, such as testing animals to be imported or testing suspect animals that are AGID-negative.

Future research using the PCR in BLV diagnosis could be directed towards early confirmation of infection in young calves in situations where the AGID test results could be confounded by colostral antibody. In addition, detection of BLV in milk samples by the PCR would be useful because it is often more convenient for dairy producers to collect milk samples than blood samples. In these 2 situations, the PCR has considerable potential utility.

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